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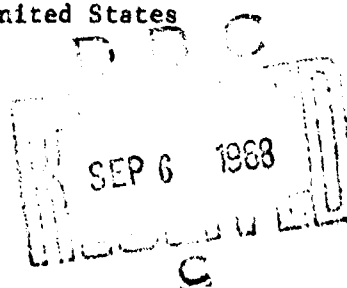
TRANSLATION NO. 1362

DAAA-13-

DATE: 5 MAY 1965

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STUDIES ON THE POLYSACCHARIDE ANTIGENS OF BRUCELLA

Following is a translation of an article by M.A. Fuks and C.A. Serpa, prepared with the aid of the National Research Council (Conselho Nacional de Pesquisas), in the Portuguese-language publication Anais de Microbiologia (Annals of Microbiology), Microbiology Laboratory, National School of Pharmacy, University of Brazil, Part A, No. 10, Rio de Janeiro, 1962, pages 63-78.⁷

I. Physical, Chemical and Physicochemical Determinations

The determination of the antigenic structure among the members of the Brucella genus has been studied by various writers, by means of immunological or chemical processes; however, there are, up to the present time, several problems without a definite solution.

When one reviews what has been written on the subject, it is seen that Burnet (1922) isolated a material that showed antigenic activity, by using filtrates of a Brucella culture.

One of the first studies on Brucella antigens was made by Schoenholtz and Myer (1927), in which these writers obtained a bacillary extract, called abortin, by freezing and unfreezing the cells. This material, which also contained nucleoprotein, showed allergizing activity.

When Wilson and Miles (1932) studied the antigenic differences between the Brucella species, they postulated the existence of two antigens, A and M, which varied quantitatively between the three species.

Favilli and Biancallani (1932), while working with specimens of Brucella melitensis and Brucella abortus, isolated a serologically active polysaccharide from the first species; however, they did not succeed in isolating an active

substance from *Brucella abortus*. The isolated material contained 4.5% N₂, gave positive reactions for uronic acid and negative reactions for pentoses.

Huston and colleagues (1934) isolated a polysaccharide fraction from *Br. suis*, *Br. abortus* and *Br. melitensis*, called polysaccharide C. in addition to proteins and nucleoproteins. Subsequently, the polysaccharide was purified, being divided into two components, C-I and C-II, with the first one being non-dialyzable and the second one easily dialyzable. Both were levorotatory, however, after hydrolysis, they became dextrorotatory; they gave a positive Mordoch reaction and negative tests for proteins. Those substances were inactive when tested in precipitation and fixation of complement reactions. Likewise, they had no allergizing or toxic activity.

Topping (1934) obtained material of a polysaccharide nature, which was active in precipitation and agglutination reactions.

Higginbotham and Healtman (1936) also isolated antigenic material that contained active polysaccharides in its structure is serum precipitation tests.

Lisbonne and Monnier (1936) isolated, by utilizing the method of Biovin and Mesrobianu (extraction with trichloroacetic acid), a glucidic-lipoprotein antigen, active in serum precipitation tests.

Pennel and Huddleson (1937) also conducted research on the antigenic components of *Brucella*. Subsequently, the same writers (1938a, 1938b) continued their studies on those antigens and determined that they showed similarity with the antigens of Biovin and Mesrobianu isolated from other Gram negative germs. By using quantitative precipitation methods, it was also observed that the substances isolated from three species of *Brucella* are not identical, since they can be differentiated serologically. They also report that although the "endo-antigens" of *Br. abortus* and *Br. suis* are very similar, although not reacting identically, they would make their serological differentiation possible in that way.

Morales-Otero and Gonzales (1938), while analyzing their material which was of proteinic nature and also contained polysaccharide varying from 1 to 1.5%, observed activity in serum precipitation tests, fixation of complement, as

well as allergic behavior in previously inoculated guinea pigs.

Roman (1938) and Renoux (1939), while working with an antigen isolated by means of the Boivin and Mesrobianu method, report good results in immunization against infection by Br. melitensis in guinea pigs. Nevertheless, Stahi and Hamman (1941) did not succeed in confirming the results of Roman and Renoux.

Huddleson (1942) obtained from living cells a hydro-soluble, labile antigen, capable of creating an immune state.

Morales-Otero and Pomales-Lebron (1943) succeeded in differentiating the species of Brucella by means of the precipitation reaction, utilizing extracts obtained after treating with formamide (Fuller's method). They ascertained that all the extracts of Br. abortus and Br. suis produce a ring of precipitation (Uhlenhuth's test) with anti-abortionus and anti-suis serums, but not with anti-melitensis serum. By using the homologous Br. melitensis system, they also obtained a positive reaction.

Parnas and Mierzejewski (1957) isolated antigenic fraction of the three species of Brucella, by studying their composition in amino acids, as well as the component sugars. Those fractions were active in agglutination, fixation of complement, passive hemagglutination reactions and allergic tests.

Leon and Cano (1958) isolated a polysaccharide from Br. melinensis /sic; should read melitensis/, after treatment with distilled water. It behaved like a complex hap-tene. When that material was treated with aluminum phosphate, it proceeded to behave like a complete antigen, producing precipitant and complement fixing antibodies, also increasing their resistance to lethal doses of Brucella, which was also observed by means of an increase in the opsonocytophagic index. Meanwhile, they did not observe allergic reactions in animals inoculated with Brucella.

Gary and colleagues (1958) described a levorotatory polysaccharide, which, after hydrolysis, contained only glucose in its molecule.

Recently, Olitzki (1959, 1960) and Olitzki and Sulitzeanu (1958) conducted research on the antigenic structure of *Brucella*. Summarizing their results, it is observed that at least six antigenic fractions can be found, and, in certain circumstances, even ten antigenic fractions have been described, by using for that purpose the immunoprecipitation method in agar gel, in accordance with Ouchterlony's method.

Carrere and colleagues (1958) made studies similar to those made by Olitzki and his colleagues, using both preparations obtained by trituration of the cells or by ultrasonic treatment, and preparations obtained after treating with trichloroacetic acid, determining, nevertheless, only one line of precipitation, using for this purpose the methods of Ouchterlony and Scheidegger. In those experiments, they also used material isolated from species of *Br. melitensis*, *Br. abortus* and *Br. suis*. They determined that the extracted antigens are identical only when obtained by trituration of the cells or after ultrasonic treatment. Nevertheless, the glucidic-lipoproteinic antigens of *Br. melitensis* and *Br. abortus* are identical, while the one isolated from *Br. suis* is antigenically different.

Barber and colleagues (1962) report the isolation of a polysaccharide fraction joined to nucleoprotein, which probably corresponds to the M antigen of Wilson and Miles, by using sodium desoxycholate. This substance does not display the behavior of a complete antigen, but it is capable of causing allergic reactions. By means of the gel-precipitation reaction on agar, they confirmed the investigation by Olitzki (1960).

As has been seen, there is much discrepancy in the studies found in writing on the subject. Besides this, the chemical structure of the *Brucella* antigens in a general way, and especially of the carbohydrate antigens, has been little studied.

In earlier study (Serpa and Fuks, 1960), the occurrence of crossreactions between the polysaccharide of *Brucella abortus* and of the tuberculosis bacillus was determined. It was in view of those results that we undertook the present research, attempting to determine the physical, chemical and physicochemical constants of the polysaccharide in question, in order to compare its structure with that of the polysaccharide of the previously studied (Fuks, 1958) tuberculosis bacillus, attempting in this way throw light on the possible mechanism for the

occurrence of those reactions.

Material and Methods

Polysaccharide Antigen: It was obtained on the basis of Brucella abortus, strain B99, using a modified Fuller's method (Fuks and colleagues, 1952-1953) to obtain the polysaccharide fraction. The mass of germs cultivated in an agar-tryptose medium was killed by heat and washed in a 0.85% saline solution three times, being finally centrifuged at a high speed (10,000 rpm for 15 minutes).

Physical, chemical and physiochemical determinations:

- a. Specific rotatory power: An aqueous solution of polysaccharide in a concentration of 100 mg% and a 10 cm pipe were used.
- b. Examination for glycogen: This was done with a Lugol solution using a concentrated solution of polysaccharide. The appearance of a reddish-brown color was considered as a positive result.
- c. Examination for nucleic acid: Dische's test, described in Kabat and Meyer (1948) was performed, using cysteine and sulfuric acid. In a positive reaction, a rose color appears.
- d. Examination for proteins: The biuret test was made and dyeing was with a solution of bromophenol blue.
- e. Examination for lipids: 0.1 ml of a concentrated solution of polysaccharide was deposited on Whatman filter paper No. 1, and, after it had dried, it was exposed in positive cases. Dyeing with a solution of Sudan Black BV was also used.
- f. Examination for phosphate: This was done in accordance with the Hanes and Isherwood (1949) tests and Feigl's (1954) spot-test.
- g. Examination for inositol: Scherer's test was made in accordance with the indications described in Polonowski (1952).

h. Presence of carbohydrates: Molisch's reaction was performed, using a recent solution of alpha-naphthol and concentrated sulfuric acid.

i. Presence of pentoses: This was determined by performing Bial's test in accordance with the method described in Hawk and colleagues (1954).

j. Presence of free reducing sugar: Horrocks's (1949) method was carried out, using a concentrated solution of polysaccharide.

k. Chromatographic analysis: 1) Hydrolysis of the polysaccharide: Hydrolysis was accomplished with normal hydrochloric acid, in a closed test-tube, by heating it at 85°C for 24 hours. This was followed by neutralization with sodium carbonate. A 4% solution of polysaccharide was used for this process. 2) Method used: Ascending chromatography in Pyrex vat, 60 cm long by 29 cm wide was utilized. 3) Chromatographic paper used: Whatman No. 1 paper was used in sheets 46 cm by 28 cm, with the hydrolyzed polysaccharide and the known sugars being deposited at intervals of 2.5 cm between them. 4) Mixture of solvents: A mixture of n-butanol (67 ml), acetic acid (23 ml) and water (10 ml), prepared 24 hours before, was used for saturating the chromatographic chamber. 5) Developers: Various developers were used, namely: a mixture of benzidine, acetic acid, ethyl alcohol (Horrocks, 1949), triphenyltetrazolium chloride, 2%, and sodium hydroxide (Wallenfels, 1950), R. antrona (Block, 1958) and Ehrlich's reagent, for glucosamine (Block, 1958).

l. Densimetry: We used the filter-weighing method, polysaccharide in a concentration of 1 g% and temperature 25°C.

m. Viscosimetry: The Ostwald viscosimeter was used and a solution of polysaccharide in a concentration of 1 g%. The temperature used was 23.5°C

n. Dialysis: Test was made, using polysaccharide in a concentration of 0.6 mg in 10 ml of saline, for 48 hours, with the saline being replaced every 10-12 hours.

o. Ultracentrifugation: The Beckman analytical ultracentrifuge was used and a 1% solution of polysaccharide in distilled water and 65,000 rpm.

p. Electrophoresis on paper: A power supply with a

450 volt capacity was used and a 3% solution of polysaccharide, for 20 hours, utilizing a volume of 0.05 ml, a current of 15 mA/cm and room temperature. The periodate method (Block, 1958) was used as a developer and simple absorption with methylene blue in an alcohol solution (Block, 1958).

q. Spectrophotometry: The Beckman spectrophotometer was used and an aqueous solution of polysaccharide antigen in a concentration of 0.5%.

r. Immunoelectrophoresis: This was performed, using 1% purified agar. veronal buffer pH 8.6 \pm 0.1, current of 0.8 mA/cm for a period of 12 hours, after which hyperimmune serum was added, with the reading taken 24 hours later.

Results

The analysis made on the material obtained from Br. abortus B99 with formamide showed that it is a polysaccharide, optically inactive, showing as a contaminant traces of glycogen and nucleic acid (deoxyribonucleic). It does contain proteins, since the biuret test and dyeing with bromophenol blue gave negative results. Likewise, it does not contain lipids or inositol.

The Bial's test was positive, indicating that it contains pentose. The Molisch's test was strongly positive.

It does not contain free reducing sugar, but does contain a small amount of phosphates (perhaps caused by the nucleic acid).

The chromatographic analysis revealed the presence of the following sugars: deoxyribose -- which could possibly come from the nucleic acid -- (12.5%), glucose -- due perhaps to the glycogen -- (11.3%), glucuronic acid (51.7%), and glucosamine (11.0%).

The viscosimetry made with Brucella polysaccharide, using a 1% solution in distilled water and the Oswald viscosimeter and a temperature of 23.5° C, revealed the following results: relative viscosity 1.1092 and absolute viscosity (η) 1.0260 centipoise.

The polysaccharide in question is not dialyzable and when subjected to spectrophotometry showed absorption in

the band from 240m μ to 270m μ , that is, in the ultraviolet band, possibly due to the contaminating nucleic acid.

The polysaccharide subject to ultracentrifugation revealed homogeneity, which was also observed with electrophoresis on paper.

The data that we have just presented are outlined in Tables I and II.

Table 1

Chemical Determinations Made with the Polysaccharide
Antigen of Brucella Abortus B99,
Isolated with Formamide

Nature of Examination	Method used	Results
Glycogen	Treatment with Lugol	Traces
Nucleic acids	Dische's test	Weakly positive
Proteins	Biuret test	
	Bromophenol blue	Negative
Lipids	Osmic acid test	
	Sudan Black BV	Negative
Phosphates	Hanes and Isherwood method;	
	Feigl's spot-test	Positive
Inositol	Scherer's test	Negative
Pentoses	Bial's test	Weakly positive
Carbohydrates	Molisch's test	Positive
Free reducing sugar	Horrock's test	Negative

Table II

Physical and Physicochemical Determinations Made
with the Purified Polysaccharide Antigen
of Brucella Abortus B99, Obtained
with Fuller's Method

Test Used	Apparatus Used	Concentration	Temp. at which test was made	Result
Viscosimetry	Oswald viscosimeter	1 g% in distilled water	23.5°C	Relative viscosity: 1.1092 Absolute viscosity: 1.0260 cp.
Spectrophotometry	Beckman spectrophotometer	0.5 g%		Absorption in the 240m μ to 270 m μ band (nucleic acid as contaminant)
Ultracentrifugation	Beckman analytical ultracentrifuge	1 g% in distilled water	room	One single band (homogeneous material)
Electrophoresis on paper. Developer: periodate, [illegible] and starch, alcoholic methylene blue	Domestically manufactured power supply 450 V potential	3 g% in distilled water, 0.05 ml. 0.15 mA/cm, 20 hours	room	One single area with low mobility
Chromatographic analysis: Solvents: n-butanol, acetic acid water (67:10 ml) Developers: a. Morrocks b. Wallenfels c. Antrona d. Ehrlich	Glass vat, 60 cm X 29 cm wide		room	Deoxyribose (which could come from the nucleic acid), Glucose (due perhaps to the glycogen) Galactose, Glucuronic acid, Glucosamine

Polarimetry	Laurent Polarimeter	c = 100 mg, 26° t = 100 <u>/Illegible/</u>	26° $\frac{[\alpha]_D}{c} =$ inactive
Continuous analysis		6 mg% 0°C	Non-dialyzable

Discussion

As was seen in the introduction to this study, several writers have studied the antigenic structure of the germs belonging to the genus Brucella, with, on the other hand, numerous points of discussion among the writers.

In the first studies found, the authors are not concerned with the smooth or rough phase of the colony; hence several of those studies are not as reliable as might be desired.

Other writers (Burnet, 1922; Schoenholtz and Meyer, 1927) were concerned with isolating substances from Brucellas, observing their antigenic activity or allergic properties.

Wilson and Miles (1932), in their study of the components of germs of the genus Brucella, postulated the existence of two antigens, but they could not be isolated.

With the studies by Favilli and Biancallani (1932), we have the description of a polysaccharide isolated from Brucella melitensis, giving a reaction for uronic acid and absence of pentose. After hydrolysis, they also found the presence of glucose and galactose.

Huston and colleagues (1934) determined, by studying a polysaccharide isolated from the three species, that this substance could be broken down into two fractions, C-I and C-II, both of which give the Molisch reaction and negative tests for proteins. Nevertheless, these fractions were inactive in tests for precipitation, fixation of complement, allergic activity, and they also are atoxic.

Meanwhile, Topping (1934) described a polysaccharide fraction that was active in a precipitation test and that also was observed by Higginbotham and Healtman (1936) in the material isolated by them.

Lisbonne and Monnier (1936) isolated a glucidic-lipoprotein antigen by extraction with trichloroacetic

acid, which was active in precipitation tests and which was also observed by Pennell and Huddleson (1937, 1938a, 1938b). These same authors also observed that it was possible to show difference between the antigens of the three species by using quantitative precipitation methods. It is to be emphasized that Pennell and Huddleson determined that materials isolated for Brucella suis and Br. abortus are very similar.

Material of a proteinic nature, containing polysaccharide as a contaminant in a proportion of 1 to 1.5%, active in precipitation and fixation of complement tests and showing allergic behavior, was described by Morales-Otero and Gonzalez (1938)

Precipitation reaction was obtained by Morales-Otero and Pomales-Lebron (1943), by using an antigen extracted by treating with formamide (Fuller's method); those authors also reported that it is possible to determine differences between the Brucella species. Those writers observed, likewise, that extracts of Br. abortus and Br. suis give cross-reactions between themselves, while the precipitation reaction in the Br. melitensis system appeared only in the homologous system.

When Parnas and Mierzejewski (1957) studied the antigenic fractions of the three species of Brucella, they determined that it was possible to obtain proteinic and polysaccharide antigens, capable of reacting in fixation of complement, agglutination, passive hemagglutination reactions and capable of producing allergic reactions.

They determined that the amino acids present were the following: leucine, isoleucine, phenylalanine, valine, methionine, proline, tryptophan, tyrosine, alanine, threonine, lysine, glycocholic acid, serine, glutamic acid, aspartic acid, cystine, arginine, and histidine; and as component sugars glucosamine, galactose, glucose, mannose, fructose, arabinose, xylose, uronic acid and another unidentified sugar.

Leon and Cano (1958) isolated a polysaccharide fraction from Br. melitensis, which behaved like a complex hapten, which, when treated with aluminum phosphate proceeded to behave like a complete antigen, stimulating the production of precipitant and complement fixing antibodies.

Gary and colleagues (1958) described a levorotatory polysaccharide, which is a polymer of glucose.

Olitzki (1959, 1960) and Olitzki and Sulitzceanu (1958) conducted research on the antigenic structure of Brucella, describing at least six antigenic fractions and, under certain conditions, even ten antigenic fractions were found by using the gel precipitation method in accordance with Ouchterlony. Meanwhile, when Carrere and colleagues (1958) made studies similar to those made by Olitzki and his collaborators, using preparations obtained by trituration of the cells or ultrasonic treatment, and after treating with trichloroacetic acid, they determined only the appearance of one single line of precipitation. Moreover, those last-mentioned authors report that the glucidic-lipoproteinic antigens differ from the ones extracted by the other two methods and that the glucidic-lipoproteinic antigens of Br. melitensis and Br. abortus are identical serologically, while the one isolated from Br. suis is antigenically different.

Barber and colleagues (1962), meanwhile, confirmed Olitzki's (1960) studies and they also report the isolation of a carbohydrate component joined to a nucleoproteinic fraction which probably corresponds to substance M of Wilson and Miles.

The polysaccharide antigen isolated by us, also by means of formamide, containing traces of glycogen and nucleic acid, shows behavior like a complex hapten (Serpa and Fuks, 1962). It differs from the polysaccharide isolated from the tuberculosis bacillus in several respects, namely: specific rotatory power, since, while the one isolated from Brucella is inactive, the one from the tuberculosis bacillus has a $[\alpha]_D^{25} = 86^\circ$; viscosimetry, being less viscous than the polysaccharide of the tuberculosis bacillus, densimetry and chromatographic analysis, in which we see that while certain sugars are not found in the polysaccharide isolated from the tuberculosis bacillus, others are common, such as galactose and glucosamine, differing nevertheless in their proportions, with the occurrence of abstraction of the glucose, which could come from the glycogen, and deoxyribose, which could be produced by the nucleic acid.

The polysaccharide isolated from Brucella, just like the one from the tuberculosis bacillus, does not contain protein, lipids or inositol, gives positive Gial and Molisch reactions, is non-dialyzable, with a maximum absorption in the 240 $m\mu$ to 270 $m\mu$ band. Its homogeneity was tested by

the analytical ultracentrifuge, electrophoresis on paper and immunoelectrophoresis.

When the material in question is compared with the data found in the literature, it can be related to the material found by Favilli and Biancallani (1932), Parnas and Mierzejewski (1957), Dubrovskaja and colleagues (1958), with regard to the presence of uronic acid, Huston and colleagues (1934) with regard to what he says concerning dialysis, but differing from this one in that the polysaccharide studied by us is highly active in precipitation tests, which has also been described by various writers (Topping, 1934; Higginbotham and Healthman, 1936; Lisbonne and Monnier, 1936; Pennell and Huddleson, 1937, 1938; Morales-Otero and Pomales-Lebron, 1943; Olitzki, 1959, 1960; Olitski and Sulitzeanu, 1958; Carrere and colleagues, 1958; Bogomolova, 1957).

It should also be mentioned that the composition of the sugars in our material is similar to that observed by Parnas and Mierzejewski (1957), in the same way as in the capability of the isolated polysaccharide to be able to fix itself on erythrocytes making their utilization possible in tests for passive hemagglutination.

Finally, it seems that the mechanism for the occurrence of cross-reactions between the polysaccharides of Brucella and of the tuberculosis bacillus could stem from the coexistence of the galactose and glucosamine fractions. Meanwhile, research on the matter is in progress.

Summary

A polysaccharide fraction isolated from Br. abortus B99, in smooth phase, by means of the formamide method (Fuller's method) is analyzed in the present paper, and an attempt is made to compare its properties and chemical and structure with the polysaccharide of the tuberculosis bacillus isolated under the same conditions and studied earlier (Fuks, 1958).

The polysaccharide isolated from Brucella differs from the polysaccharide of the tuberculosis bacillus with regard to densimetry, viscosimetry, specific rotatory power and presence of uronic acid.

With a chromatographic analysis, differences were observed with regard to the proportions of the component sugars,

with galactose and glucosamine occurring as common sugars with abstraction of glucose occurring (which could be caused by the glycogen found as a contaminant in traces).

The polysaccharide in question has the behavior of a complexe haptene, does not contain protein, lipids or inositol, is not dialyzable, is homogeneous when tested by the analytical ultracentrifuge, electrophoresis on paper and immunoelectrophoresis.

Summary

In the present paper a polysaccharide fraction isolated from a smooth *Brucella abortus* B99 strain by the formamide technique (Fuller's technique) was analysed. Its properties and chemical constitution have been compared with those of the polysaccharide fraction of the tubercle bacilli isolated under the same conditions and studied earlier (Fuks, 1958) because of the occurrence of cross reactions between them.

The density, viscosimetry, optical rotation are different in both polysaccharides. *Brucella* polysaccharide contains uronic acid.

The polysaccharide fraction has a behavior of a complex hapten, it does not contain protein, lipids or inositol. It does not dialyse and showed homogeneity when submitted to analytical ultracentrifuge, paper electrophoresis and immunoelectrophoresis techniques.

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